Investigating the Use of *Ralstonia eutropha*'s H_2-Sensing Pathway in a Heterologous Biological H_2-Sensing Reporter

by

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ABSTRACT

Increasing interest in alternative fuels has driven increasing interest in biologically derived hydrogen, such as that produced by phototrophic microorganisms like cyanobacteria and green microalgae. The goal of this study was to test whether the hydrogen-sensing pathway of the soil bacteria *Ralstonia eutropha* could drive expression of a luciferase in a hydrogen-dependent manner so as to form a heterologous biological hydrogen-sensing reporter. Plasmids containing the *hox* and *hyp* clusters of genes reported to be necessary for hydrogen sensing in *R. eutropha* were introduced into *Escherichia coli*. However, the lack of bioluminescence in response to hydrogen demonstrated that those genes were not sufficient to serve as a hydrogen-sensitive transcription regulator in *E. coli*. This indicates that there is some factor not currently described that is necessary for the system's proper functioning in *R. eutropha*. The idea of a heterologous hydrogen-sensing reporter could be revisited once the pathways have become more fully characterized.

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CHAPTER ONE: Introduction

There is currently a great interest in developing alternative energy sources to replace the nonrenewable fossil fuels. Potential alternative energy sources face multiple considerations, such as efficiency, renewability, and impact on the environment. Many of the alternative energy sources being developed currently fall short in one or more of these areas.

Hydrogen has great potential as an alternative energy source because of its efficiency and negligible impact on the environment when burned. With an energy content of 122 kJ/g, which is 2.75 times greater than hydrocarbon fuels (Argun *et al*. 2008), and no CO_2 output when burned, hydrogen's main shortcoming at the present is an inexpensive and renewable means of production.

Biological hydrogen production has been put forward as one means of renewably obtaining the gas. Hydrogen is an important component of the microbial world, with 300 teragrams of hydrogen being produced and consumed by microbes each year (Thauer 2011). Many nitrogen-fixing phototrophs such as cyanobacteria and green algae produce hydrogen as a byproduct of their nitrogen fixation. Additionally, hydrogen can be produced by hydrogenase enzymes or by splitting water during photosynthesis (Azwar *et al*. 2014). If microbes such as

these could be cultured and the hydrogen collected in large volumes, this would be an inexpensive and renewable form of hydrogen production since the energy is provided by the sun and the machinery self-assembling.

Hydrogenases

Microbes across all three domains use enzymes called hydrogenases to capture some of the energy stored in molecular hydrogen. While our currently existing fuel cells use the expensive and rare platinum as a catalyst to oxidize H_2 with O_2 as the electron acceptor, microbial hydrogenases use nickel and iron atoms at their catalytic centers. Understanding hydrogenases in greater detail could lead to the development of much cheaper H_2/O_2 fuel cells in the future. Some hydrogenases are also capable of producing hydrogen rather than just oxidizing it.

Hydrogenases are a class of enzymes that catalyze the reversible reaction $H_2 \leftrightarrow 2H^+ + 2e^-$ (Constant *et al*. 2011). While most of the currently known hydrogenases are capable of running the reaction in both directions *in vitro,* the needs of the host organism usually commit each hydrogenase to either the production or utilization of hydrogen *in vivo* (Vignais *et al*. 2001).

The largest group of hydrogenases is the [NiFe]-hydrogenases (Vignais *et al*. 2001), which were originally isolated from bacteria (Adams *et al*. 1981). Some

archaea were later found to also possess [NiFe]-hydrogenases (Graf & Thauer 1981), though they have never been found in eukaryotes (Vignais *et al*. 2001). Originally divided into four major groups, a fifth group of [NiFe]-hydrogenases has recently been discovered (Constant *et al*. 2011). The basic structure shared by all [NiFe]-hydrogenases is a heterodimer composed of a large subunit and a small subunit (Przybyla *et al*. 1992), which appear to have evolved together (Vignais *et al*. 2001).

Group 1 hydrogenases are termed "Membrane-bound Uptake [NiFe]-hydrogenases" (Vignais & Billoud 2007), which are characterized by a signal peptide that results in the fully folded heterodimer being exported to the membrane through the twin-arginine translocation (Tat) pathway (Vignais & Billoud 2007). As their name implies, these uptake hydrogenases are used to oxidize molecular hydrogen in order to reduce some electron acceptor. The cell ultimately captures energy through the generation of a proton motive force. Some organisms containing Group 1 [NiFe]-hydrogenases include *Escherichia coli, Ralstonia eutropha, Wolinella succinogenes, Aquifex aeilicus, Hydrogenobacter thermophilus, Thiocapsa roseopersicina,* multiple members of the *Desulfovibrio* genus, and the archaeon *Methanosarcina mazei* (Vignais & Billoud 2007).

Group 2 [NiFe]-hydrogenases differ from Group 1 in that they are cytoplasmic rather than membrane-bound. They are further divided into two subcategories, cyanobacterial uptake [NiFe]-hydrogenases and molecular hydrogen sensors (Vignais *et al*. 2001). The occurrence of cyanobacterial uptake [NiFe]-hydrogenases (Group 2a) correlates to the occurrence of nitrogenases and they are upregulated by conditions conducive to nitrogen fixation (Appel & Schulz 1998). Fixing relatively chemically inert atmospheric nitrogen to the much more bioavailable ammonium evolves molecular hydrogen, so this would allow nitrogen-fixing cyanobacteria to recapture some of the energy lost to otherwise useless dihydrogen formation. The best-studied hydrogenases of Group 2a are found the cyanobacterial genera *Nostoc* and *Anabaena* (Vignais *et al*. 2001). Group 2b is composed of regulatory hydrogenases used to upregulate other hydrogenases in the presence of dihydrogen. This allows organisms to only produce large amounts of hydrogenase when it would be beneficial. Group 2b hydrogenases are well characterized in *R. eutropha, R. capsulatus, T. roseopersicina, Rhodopseudomonas palustris,* and *Bradyrhizobium japonicum* (Vignais & Billoud 2007). Unlike most hydrogenases, these sensor hydrogenases are not sensitive to oxygen. It was confirmed through mutagenesis experiments that

oxygen is physically incapable of reaching the active site of the enzyme, allowing the sensors to function in lower concentrations of hydrogen (Buhrke *et al*. 2005).

Group 3 [NiFe]-hydrogenases can be divided into four subcategories. However, they all share certain characteristics: they are cytoplasmic rather than membrane-bound, they are composed of multiple subunits of different kinds (heteromultimeric), and they are considered bidirectional or reversible (Vignais & Billoud 2007). Group 3a [NiFe]-hydrogenases are found in obligate anaerobic methanogenic archaea. The group can be even further divided into two more groups, one that uses H_2 and CO_2 or formate as substrates, and another that uses C1 compounds such as methanol or methylamines (Deppenmeier *et al*. 1999). Group 3b [NiFe]-hydrogenases are found in hyperthermophiles such as *Pyrococcus furiosus* and are tetrameric, while Group 3c [NiFe]-hydrogenases are able to reduce methyl viologen (Vignais *et al*. 2001). The Group 3d [NiFe]-hydrogenases use the electrons produced by the splitting of H_2 to reduce NAD^+ and were first isolated from *Ralstonia eutropha* (Schneider & Schlegel 1976). In addition to their Group 2a hydrogenases that are upregulated under nitrogen-fixing conditions, cyanobacteria have bidirectional Group 3d hydrogenases that are constitutively expressed (Schmitz *et al*. 1995). They are also believed to be present in species that do not fix nitrogen (Vignais *et al*. 2001).

Group 4 [NiFe]-hydrogenases are enzymes with at least six subunits associated with the membrane and produce molecular hydrogen. The first hydrogenase from this group to be studied in depth was *E. coli*'s hydrogenase 3 (Vignais *et al*. 2001). However, most Group 4 hydrogenases discovered so far have been found in archaea, including *Pyrococcus furiosus* (Vignais & Billoud 2007).

Group 5 [NiFe]-hydrogenases have been discovered much more recently. First discovered in *Streptomyces* species and reported in 2010, these hydrogenases show a very high affinity for hydrogen and are able to oxidize H_2 at atmospheric levels (Constant *et al*. 2011). Bacteria containing these hydrogenases absorb 50 teragrams of H_2 from the atmosphere annually (Schafer *et al*. 2013), which is 80% of the hydrogen lost from the atmosphere every year (Constant *et al*. 2011). Growth of *Mycobacterium smegmatis* was shown to be significantly reduced when its Group 5 hydrogenases were inactivated, and both the structural and accessory genes for the hydrogenases were upregulated upon growth on poor carbon sources; while this is similar to *R. eutropha's* hydrogen-indpendent upregulation of hydrogenases when grown on poor carbon sources, Berney & Cook (2010) suggest this means the high-affinity nature of the Group 5

hydrogenases allows *M. smegmatis* to "scavenge" molecular hydrogen from the atmosphere.

Ralstonia eutropha

The betaproteobacterium *Ralstonia eutropha* is able to grow on CO_2 as its sole carbon source and molecular hydrogen as its sole electron donor, which it does through the use of two hydrogenases: a soluble cytosolic hydrogenase (SH), which reduces NAD^+, and a membrane-bound hydrogenase (MBH), which is linked to a *b*-type cytochrome (Lenz & Friedrich 1998). The genes encoding the subunits of these two hydrogenases are located in two different operons on the megaplasmid pHG1, each also containing genes encoding associated accessory proteins. Production of these hydrogenases is regulated at the level of transcription with input from a hydrogen-sensing pathway and a poorly understood "energy-responsive factor" (Buhrke *et al*. 2004).

The hydrogen-sensing system of *R. eutropha* consists of three proteins encoded by four genes. The proteins HoxA, an NtrC-like activator, and HoxJ, a histidine kinase, form a standard two-component regulatory system. However, in contrast to the usual relationship in these systems, the phosphorylation of HoxA results in its inactivation. Thus, the HoxJ protein functions as a repressor. In the absence of molecular hydrogen, HoxJ inactivates HoxA, halting

transcription of the hydrogenase genes. The genes *hoxB* and *hoxC* encode the small and large subunits, respectively, of a third hydrogenase, called the regulatory hydrogenase (RH). This hydrogenase carries out its hydrogen-splitting reaction at a rate two orders of magnitude lower than the other hydrogenases (Burhke *et al*. 2004) and functions as a hydrogen sensor. As the RH splits H_2, it is believed that the resulting free electrons are transferred to a cofactor bound to HoxJ, stopping it from inactivating HoxA. This allows HoxA to bind to the promoters for the SH and MBH operons to start transcription.

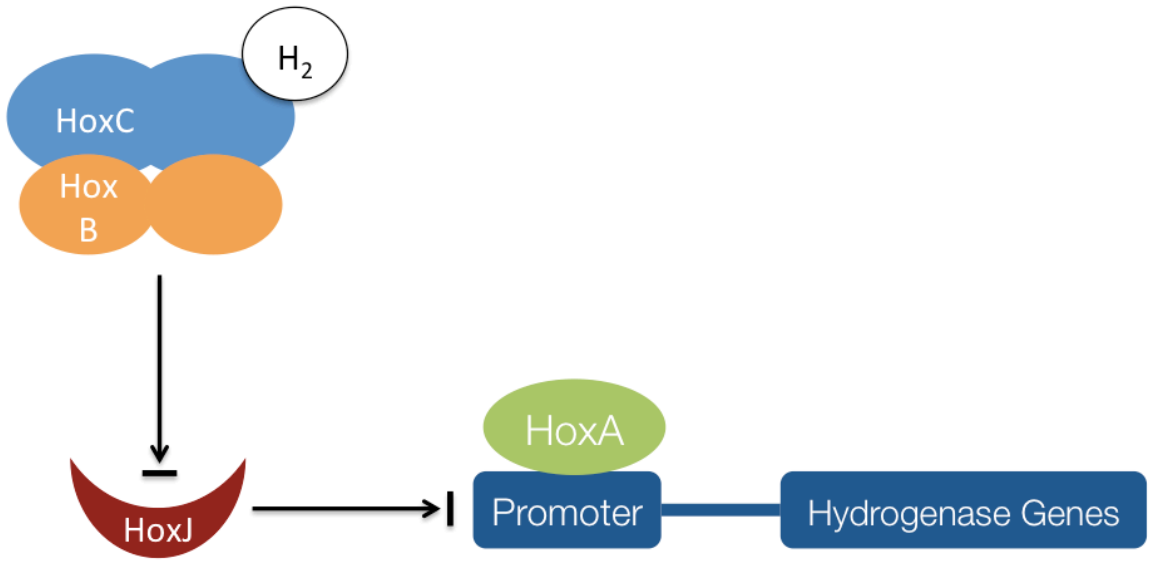

Figure 1: The Hydrogen-Sensing Pathway of *R. eutropha* – When the Hox$(BC)_2$ hydrogenase splits hydrogen, it transfers the liberated electrons to a cofactor bound to HoxJ, inactivating it. This stops HoxJ from inactivating HoxA, allowing the latter to start transcription of the hydrogenase genes.

In addition to the hydrogen-sensing pathway, transcription of SH and MBH genes is dependent in some way on the energy sources available to the cell, though this mechanism is not completely understood. Production of the hydrogenases is tightly repressed when *R. eutropha* is grown on succinate (a preferred carbon source), though this repression is relaxed when the succinate is depleted and the cells enter stationary phase (Friedrich 1982). Cells grown on succinate will tightly repress hydrogenase production even when the cells are exposed to hydrogen during growth (Lenz *et al*. 2002). Cells grown on non-preferred carbon sources such as fructose or glycerol tend to produce very low amounts of hydrogenase in the absence of hydrogen, but this changes substantially when the cells are grown in the presence of hydrogen. The starkest contrast can be seen in the MBH operon in cells grown on glycerol with and without hydrogen (Lenz *et al*. 2002). Since cells grown on a preferred carbon source will repress hydrogenase production even in the presence of hydrogen, it is implied that either a.) a factor other than HoxA is required to start transcription of the SH and MBH genes, or b.) a factor controlled by the availability of energy sources can either block HoxA's binding to its cognate promoters or inactivate it similar to HoxJ. In any case, regulation of *R. eutropha*'s hydrogenases is dependent on more than the presence or absence of hydrogen.

Unfortunately, the role that energy sources play in this system is presently not any more understood than presented here (Greening & Cook 2014), so it is unclear what effect, if any, would be had on heterologous expression of the hydrogen-sensing system.

Transcription of the SH and MBH operons is dependent on the alternative sigma factor σ^{54} (Schwartz *et al*. 1998). Genes encoding σ^{54} proteins have been found in a wide variety of bacterial species, both Gram positive and Gram negative, and have been found to control expression of a wide variety of proteins (Buck *et al*. 2000). While σ^{54} proteins bind to RNA polymerase to form a holoenzyme like σ^{70} family members, there appears to be no sequence conservation between the σ^{54} and σ^{70} groups (Kazmierczak *et al*. 2005). The σ^{54} holoenzyme requires a separate activator protein, HoxA in the case of *R. eutropha*, in order to start transcription. This allows a single σ^{54} protein to be responsible for the regulation of multiple genes under many different environmental conditions (Buck *et al*. 2002). It also allows for very low levels of "leakiness" in expression due to the tightness of regulation (Wang & Gralla 1998). Since *hoxA* is found in the MBH operon, its activation begins a positive feedback loop, leading to quick increases in hydrogenase expression.

Successful heterologous expression of *R. eutropha*'s hydrogenases and related proteins has been very limited until recently. HoxA was expressed in *E. coli*, where it mediated relatively low-levels of expression of a reporter driven by P_{SH} or P_{MBH} as compared with *R. eutropha* (Schwartz *et al*. 1998). While early attempts at expressing catalytically active hydrogenases from *R. eutropha* in *E. coli* failed (Kleihues *et al*. 2000), including an attempt to express catalytically active RH (Bernhard *et al*. 2001), the desire for oxygen-tolerant hydrogenases for use in biofuel production spurred further research. Two separate groups, Schiffels *et al*. and Ghosh *et al*., successfully expressed catalytically active SH in *E. coli* in 2013. In both cases, it was found that expression of associated accessory proteins was absolutely necessary for producing catalytically active enzyme. These *hyp* genes are found in two clusters on pHG1, with one set located between the structural genes for the MBH and RH and the other located downstream of the SH structural genes. It was originally thought that homologs in *E. coli* might be able to fulfill the same function during heterologous hydrogenase expression, but this was found to be not the case. Furthermore, it was found that the paralogous genes in *R. eutropha* were poor substitutes for each other, resulting in hydrogen oxidation an order of magnitude lower than with the proper Hyp proteins. That is, expression of the SH in *E. coli* with the

Hyp proteins associated with the MBH was far less efficient. Full expression of catalytically active SH required co-expression of the *hyp* genes found in the same operon. Interactions between HoxA and HoxJ, along with the hydrogen-sensing system as a whole, have never been tested heterologously.

R. eutropha (strain H16, ATCC 17699) has been officially renamed a number of times. In the early 90s it was a part of the genus *Alcaligenes* before being relocated to *Ralstonia* in 1995 (Yabuuchi *et al*. 1995). It was moved to *Wautersia* in 2004 before DNA analysis reported that same year revealed the bacteria to actually be the same as the previously described *Cupriavidus necator* (Vandamme & Coenye 2004). Despite this, the *Cupriavidus necator* name has been slow to catch on in the literature and many papers discard the convention altogether or briefly acknowledge it before ignoring it. This report follows that pattern and will refer to the species as *Ralstonia eutropha* throughout.

Luciferases/Reporters

Bioluminescent organisms produce luciferases, enzymes that catalyze a chemical reaction to generate light. Luciferases are widely distributed across life, found in over 700 genera, 80% of which are marine (Widder 2010). This is not surprising as life began in the ocean and sunlight grows fainter as ocean depth increases. In clear water, light dims by an order of magnitude every 75 meters

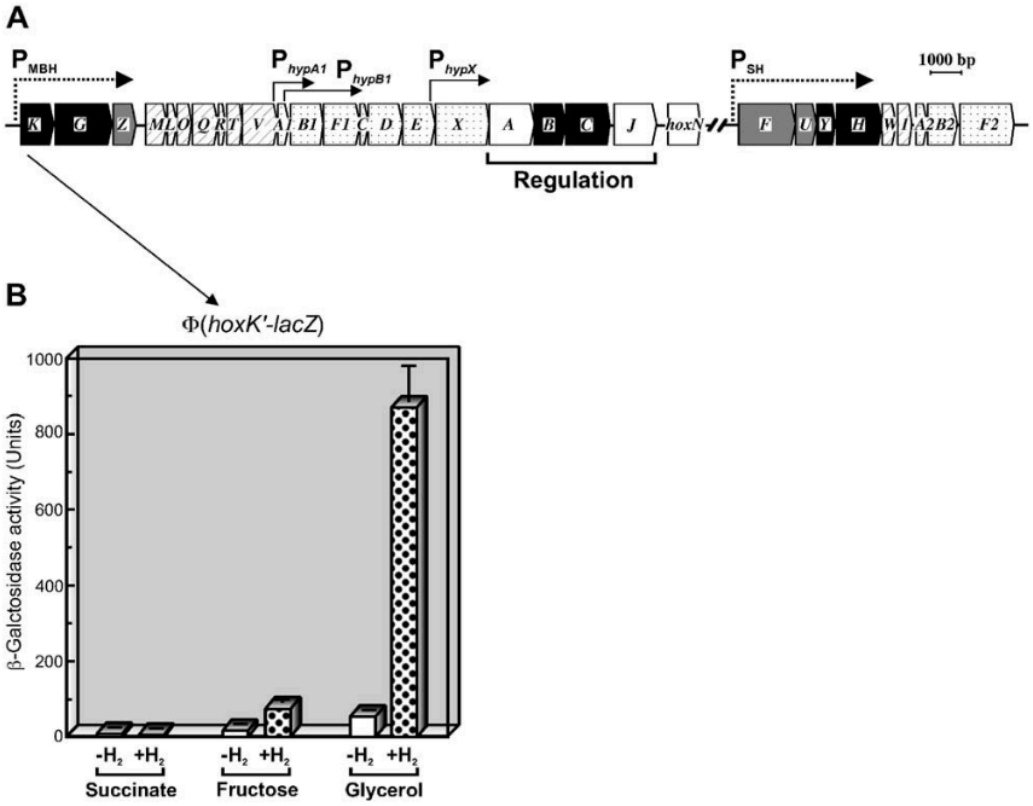

Figure 2: H_2-Dependent Expression of the Hydrogenase Regulon of *R. eutropha* (adapted from Lenz *et al*. 2002) – A) Gene order of the MBH and SH operons B) Hydrogenase expression on different carbon sources, measured with a β-galactosidase reporter.

before becoming totally extinguished at 1,000 meters (Widder 2002). This provides a distinct advantage to organisms that can produce and utilize their own light. Luciferases have long been used as a tool in molecular biology, both as reporters of endogenous gene expression and as part of heterologous gene expression systems (Gould & Subramani 1988).

Bacterial luciferase is not evolutionarily related to any other known luciferase (Fisher *et al*. 1995). The two subunits that compose the enzyme, designated alpha and beta and coded for by the genes *luxA* and *luxB*, share around 30% sequence identity depending on the strain; it is thought that the beta subunit arose as a product of gene duplication at some point before the lineages leading to present-day bioluminescent bacteria diverged (Baldwin *et al*. 1979). In addition to high sequence identity, the secondary structural topology of the alpha and beta subunits is identical (Fisher *et al*. 1995). Given that the subunits are capable of catalyzing the light reaction separately to a small extent and beta subunits will form homodimers *in vitro* (Sinclair *et al*. 1993, Sinclair *et al*. 1994), it is possible that the original enzyme was coded for by a single gene that formed homodimers and therefore contained a catalytic site on both subunits (Fisher *et al*. 1995). The alpha subunit is approximately 40 kDa in size

while the beta subunit is approximately 35 kDa; the alpha subunit has a 29 amino acid insertion at residue 259 of the beta subunit (Fisher *et al*. 1995).

Luciferases act on substrates called luciferins, though the chemistry of these molecules differs wildly between luciferases (Hastings 1996). A long chain fatty aldehyde acts as the luciferin for bacterial luciferase. Though a number of different long chain fatty aldehydes are known to be found in bioluminescent bacteria and take part in this reaction (Shimomura *et al*. 1974), it is believed that the original aldehyde used was tetradecanal due to the enzyme's affinity for it at low concentrations, though some differences in aldehyde specificity do exist between different bioluminescent bacteria (Meighen 1991). Generally speaking, as aldehyde chain length decreases, increasing concentrations of the aldehyde are required to carry out the reaction (Meighen & Dunlap 1993).

Luciferases from the beetle family are also commonly used as reporters. As stated previously, they are evolutionarily unrelated to the bacterial luciferases, and require a different substrate. Early research focused almost exclusively on the luciferase from the North American firefly *Photinus pyralis*, but luciferases of multiple colors have since been isolated from the click beetle *Pyrophorus plagiophthalamus* (Wood *et al*. 1989). Wood and his colleagues reported the discovery of four different luciferases from the click beetle: a green,

a green-yellow, a yellow, and an orange. The amino acid sequences of these luciferases are between 95% and 99% identical with each other, though they are only 48% identical to the amino acid sequence of firefly luciferase. The authors suggest these might be useful in dual-reporter systems, though that would require capability to resolve their emission spectra. Commercially available beetle luciferases have since been codon optimized for use in mammalian cells and had the peroxisome targeting sequence removed to allow large-scale localization to the cytoplasm (Contag & Bachmann 2002).

Objective/Significance

The goal of this study is to investigate the viability of combining *R. eutropha*'s hydrogen-sensing pathway with a luciferase to create a biological hydrogen-sensing reporter for heterologous expression in hydrogen-producing phototrophic microbes such as cyanobacteria or green microalgae, based on the current understanding of the pathway in the literature. This could create a high-throughput screen to identify improved H_2 producers as part of lab-directed evolution. In this plan, the genes responsible for hydrogen production, whether they're hydrogenases or part of a nitrogen-fixing pathway, would be randomly mutagenized with error-prone PCR and randomly inserted into a vector to form

a plasmid library. Cultures could either then be grown in patches on agar or in liquid media and imaged for brightness in 96-well plates.

For the purposes of this study, *Escherichia coli* will be used as the model organism for construction of this system. Since heterologous expression of the full hydrogen-sensing system from *R. eutropha* has never been tested, it will be advantageous to use a genetically tractable organism with a fast generation time and established protocols for DNA manipulation. Furthermore, an organism that produces hydrogen would not be ideal while developing the system because it would be harder to control for the level of hydrogen the cells are exposed to. If it is determined which genetic elements from *R. eutropha* are necessary for heterologous expression of the system, it can then be moved to hydrogen-producing organisms to be used as a screen.

CHAPTER TWO: Materials and Methods

Bacterial Strains

Four strains of bacteria were used for all experiments. Experiments involving cyanobacteria were carried out with *Synechococcus elongatus* PCC 7942. All cloning was performed using the *E. coli* strain Top10 from Invitrogen. Top10 was also the *E. coli* strain used for all experiments involving the testing of the hydrogen-sensing reporter. *Ralstonia eutropha* H16 (ATCC 17699) was used for endogenous testing of the hydrogen-sensing system. *E. coli* S17-1 λpir was used for conjugative transfer of plasmids to *R. eutropha*.

Bacterial Growth Conditions

Unless otherwise stated, E. coli was grown in LB medium. Antibiotic concentrations used were: ampicillin, 100 µg/mL; kanamycin, 50 µg/mL; chloramphenicol, 35 µg/mL; spectinomycin 100 µg/mL.

Cyanobacteria were grown in BG11 medium (Stanier *et al*. 1977). Antibiotic concentrations used were: spectinomycin, 40 µg/mL; chloramphenicol, 7.5 µg/mL.

R. eutropha was grown either in a modified LB medium (0.5% w/v NaCl) or a mineral salts medium (recipe in Appendix) containing 0.4% w/v glycerol (GN medium), 0.05% w/v fructose and 0.4% w/v glycerol (FGN medium), or

0.4% w/v succinate (SN medium). Kanamycin was added to a final concentration of 200 µg/mL.

Where appropriate, the following concentrations were used: agar, 1.5% w/v; IPTG, 1 mM; D-luciferin (potassium salt, GoldBio LUCK-100), 0.1 mM; decanal, 2% v/v (in mineral oil). Decanal was placed in a microcentrifuge tube cap on the agar.

Plasmid Construction

Three plasmids were constructed for use in cyanobacteria, based on the plasmids pAM1580 (Andersson *et al*. 2000) and pTrc-NSI (Xu *et al*. 2003). pAM1580 contains a promoterless *luxAB* operon and chloramphenicol resistance gene flanked by areas of homology for integration into neutral site II of *Synechococcus elongatus* PCC 7942. An 881bp region of DNA immediately before the *hoxK* gene on pHG1 was amplified with primers 1 & 2 (primer table in Appendix) with Xho I and Xba I sites on the 5' and 3' ends, respectively. This piece was assumed to contain the P_{MBH} and was inserted into the multiple cloning site of pAM1580 to drive transcription of *luxAB*. pTrc-NSI contains a *trc* promoter and spectinomycin resistance gene flanked by areas of homology for integration into neutral site I of *S. elongatus*. Primers 3 & 4 were used to amplify a 6913bp region of DNA from the beginning of *hoxA* to the end of *hoxN1* on

pHG1 with BamHI sites on either end. Due to low PCR efficiency of the large amplicon, this was subcloned into the pCR2.1 vector using a TOPO-TA Cloning Kit from Invitrogen using the manufacturer's instructions. White colonies were colony PCR screened with primers 5 & 6 to confirm the presence of the *hox* genes. The *hox* genes were then cut from pCR2.1 using BamHI and inserted into pTrc-NSI at the multiple cloning site. Primers 7 & 8 were used to confirm correct insertion orientation of the piece.

hox-pTrcNSI was used to create a *hoxJ* deletion variant. FseI and MscI were used to remove a 4080bp region beginning halfway through *hoxB* and ending a third of the way into *hoxN1*, so that the only functional protein product made is HoxA.

One plasmid was constructed for use in *R. eutropha*, based on the broad-host-range vector pBBR1MCS-2 (Kovach *et al*. 1995). The 881bp region containing P_{MBH} was amplified using primers 9 & 10 with XhoI and HinDIII sites on the 5' and 3' ends, respectively, and inserted into CBG99-basic from Promega. A 2582bp piece was cut from the resulting P_{MBH}-CBG99-basic with SacI and XbaI and inserted into pBBR1MCS-2.

Three plasmids were constructed for use in *E. coli*, two based on pTrc99A (Amann *et al*. 1988) and one based on pACYC184 (Chang & Cohen 1978). The

first pTrc99A plasmid (pTrcGlo) contains *hoxABCJN1* driven by the *trc* promoter in pTrc99A and CBG99 driven by P_{MBH} immediately following, but facing the other direction. The *hox* genes were cut from *hox*-pCR2.1 with BamHI and inserted into pTrc99A. Primers 11 & 12 were used to sequence a portion of the resulting plasmid to confirm correct insertion orientation of the *hox* genes. Primers 13 & 14 were used to amplify a 2543bp region of DNA from P_{MBH}-CBG99-basic with SbfI sites on either end. This was subcloned into pCR2.1 with the TOPO-TA Cloning Kit. A 2545bp piece was cut from P_{MBH}-CBG99-pCR2.1 with SbfI and XbaI and inserted into *hox*-pTrc99A immediately downstream of the *hox* genes. Primers 11 & 15 were used to screen for correct insertion orientation of P_{MBH}-CBG99. The resulting plasmid *hoxABCJN1*-P_{MBH}-CBG99-pTrc99A was designated pTrcGlo and is 13628bp.

pTrcGlo was used to create a *hoxJ* deletion variant. FseI and MscI were used to remove a 4080bp region beginning halfway through *hoxB* and ending a third of the way into *hoxN1*, so that the only functional protein product made is HoxA. The final plasmid is 9548bp.

A plasmid was constructed based on pACYC184 that contains the *hypA1B1F1CDEX* genes from the MBH operon. Primers 16 & 17 were used to amplify a 6952bp region starting with *hypA1* and ending with *hypX*, which was

subcloned into pCR2.1 with the TOPO-TA Cloning Kit. The primers have HinDIII and BclI sites on the 5' and 3' ends, respectively; and the 5' primer contains a Shine-Dalgarno sequence and a modified *tac* promoter that's missing the repressor binding site. The *hyp* genes were cut from *hyp*-pCR2.1 with HinDIII and BclI and inserted into pACYC184. The modified *tac* promoter was designed for this study, so to confirm that the promoter correctly functions to drive transcription of the *hyp* genes, primers 18 & 19 were used to amplify CBG99 with SacI sites on either end for in-frame insertion into the *hyp*-pACYC184 plasmid 3 amino acids into *hypA1*. Transformants were screened for glowing. Primers 20 & 17 were also used to confirm the presence of *hypX* after insertion of the *hyp* genes into pCR2.1 and pACYC184, and after insertion of CBG99 into *hyp*-pACYC184. *hyp*-pACYC184 is 10974bp.

One plasmid, pTrc99A-CBG, was a generous gift from Chris Davis of the Robertson lab and was used unmodified. This plasmid contained CBG99 luciferase and a Shine-Dalgarno sequence inserted after the *trc* promoter in pTrc99A.

Conjugative Transfer

E. coli S17-1 λpir was transformed with P_{MBH}-CBG99-pBBR1MCS-2 and mixed with *R. eutropha* H16 in liquid culture at a 10:1 ratio for 4 hours. The

bacteria were then plated on M9 agar with 200 µg/mL kanamycin. S17-1 λpir contains the genes for the RP4 transfer proteins integrated into the chromosome, but it doesn't include any origin of transfer. The pBBR1MCS-2 plasmid contains an origin of transfer, but no genes for the required transfer proteins. This allows S17-1 λpir to transfer plasmids containing the proper origin of transfer, but the recipient bacteria are unable to transmit the plasmid they receive unless they otherwise possess these genes as well. As S17-1 λpir is auxotrophic, they will not grow on minimal media such as M9, selecting for the *R. eutropha*.

Experiments Involving Hydrogen

Hydrogen was produced through electrolysis of water in a Rubbermaid plastic container using baking soda as the electrolyte. The power source was a Linksys AC-DC converter outputting 12 volts and 500 milliamps. The wires were spliced to Radioshack 24 gauge, solid 2-conductor copper speaker wire. At the anode, the wire was simply coiled up and submerged. A test showed that a copper cathode disintegrated within a few minutes, so the cathode speaker wire was looped with a small piece of platinum. The point of contact was covered with caulk and hot glue to prevent contact of the copper with the electrolyte solution. A syringe with the plunger removed was placed over the anode and

filled with the electrolyte solution. As hydrogen collected in the syringe, the electrolyte solution was displaced. The hydrogen was then pulled into another syringe before being delivered. The gas was confirmed to be hydrogen by transferring it to a tube and holding it near a flame, resulting in its popping. The syringes were confirmed to be able to hold at least some hydrogen for a few days, though the hydrogen was almost always prepared fresh for the experiments. Earlier experiments used a gel electrophoresis box and TAE to produce hydrogen, though this method was considerably slower.

Both *R. eutropha* and *E. coli* were grown on their respective agars poured into the bottoms of flasks. These flasks were gassed with hydrogen and stoppered before being incubated upside-down at the appropriate temperature. It was confirmed through popping that the stoppered flasks could hold hydrogen at least overnight without substantial hydrogen loss. Cells not treated with hydrogen were either left unstoppered and incubated normally or were gassed with helium and stoppered like the hydrogen-treated flasks. In early experiments involving cyanobacteria, *S. elongatus* was grown on BG11 agar; before bacteria were imaged, the plate was held upside-down and 50mL of hydrogen was “squirted” onto it. All plates and flasks were imaged with the Bio-Rad ChemiDoc MP System.

For experiments requiring the plating of equal numbers of cells of two strains, cells were grown in successive liquid cultures overnight. Cell densities were measured with a Spectronic Genesys 5 spectrophotometer and each new culture was seeded with a proportionally different volume to achieve equal cell number.

Dark box experiments were carried out with bacteria grown on agar in armed flasks. Once the bacteria were grown, they were exposed to hydrogen, helium, or oxygen via a tube connected to the flask arm and run out of the dark box. Luminescence was measured with a Hamamatsu photomultiplier tube (HC135-01). The flasks were stoppered, but the stoppers had a single hole in the middle so that the gas could be injected without drastically increasing the pressure in the flasks.

CHAPTER THREE: Results and Discussion

Cyanobacteria

Since *R. eutropha*'s hydrogen sensing system has never been fully expressed heterologously, it was decided to move the genes described in the literature as required for the system's correct functioning to cyanobacteria to see if the system would work right away. This work would presumably be done at a later stage regardless; and if the cyanobacteria immediately glowed in a hydrogen-responsive manner, a great deal of time working with an intermediate organism would have been saved. *S. elongatus* PCC 7942 transformed with P_{MBH}-pAm1580 and *hox*-pTrc-NSI were grown in patches on BG11 agar with spectinomycin and chloramphenicol. *S. elongatus* carries homologs of most of the *hyp* genes in the MBH operon of *R. eutropha,* so it was hypothesized that these homologs could rescue any phenotypic deficiencies produced by the absence of *R. eutropha*'s *hyp* genes. Once grown, the plates were transiently exposed to hydrogen and imaged. Though this experiment was repeated multiple times, no bioluminescence was ever detected. Since it was unclear which part of the system was failing (anywhere from hydrogen delivery to any of the proteins involved in the hydrogen sensing system), it was decided to investigate the problem using the same plasmids in *E. coli*.

Bacterial Luciferase and Hydrogen Sensitivity

The two plasmids constructed for use in cyanobacteria contain the same *E. coli* origin of replication, leading to unstable maintenance when double transformation is attempted. However, one experiment was conducted using these plasmids. *E. coli* containing P_{MBH}-pAm1580 and either *hox*-pTrc-NSI or Δ*hoxJ*-pTrc-NSI were grown on agar in a flask and placed inside a dark box; hydrogen, oxygen, and air were pumped in as described in Materials and Methods, and light output was measured with a photomultiplier tube (Figure 3). In the case of all three gases, there was an immediate drop in luminescence, which rebounded fairly quickly. In the case of hydrogen and oxygen, this rebound was followed by a jump in brightness. These were roughly comparable between oxygen and hydrogen, with hydrogen sometimes greater and oxygen sometimes greater. *E. coli* with pTrc99A-CBG were grown in the same manner and exposed to hydrogen, oxygen, helium and air (Figure 3). Exposure to hydrogen led to a small increase in brightness, but exposure to oxygen led to an increase tens of times greater. Somewhat surprisingly, exposure to helium led to an increase in brightness roughly comparable to hydrogen exposure.

Critically, these results show that bacterial luciferase is unsuitable for use as a reporter in a hydrogen-sensing pathway due to an inherent sensitivity to

hydrogen. This may be due to a flavin mononucleotide used in the reaction, in which the reduced form is oxidized. Flooding the cells with hydrogen gas may allow rapid reduction of the oxidized product, leading to an increase in reactants. A luciferase not inherently as sensitive to hydrogen, such as click beetle luciferase, would be a better choice for a hydrogen-sensing reporter system. Because of this, it was decided to construct a new vector using CBG99 luciferase from Promega. To avoid the problem of multiple plasmids using the same origin of replication, it was decided to put all necessary genes on a single plasmid.

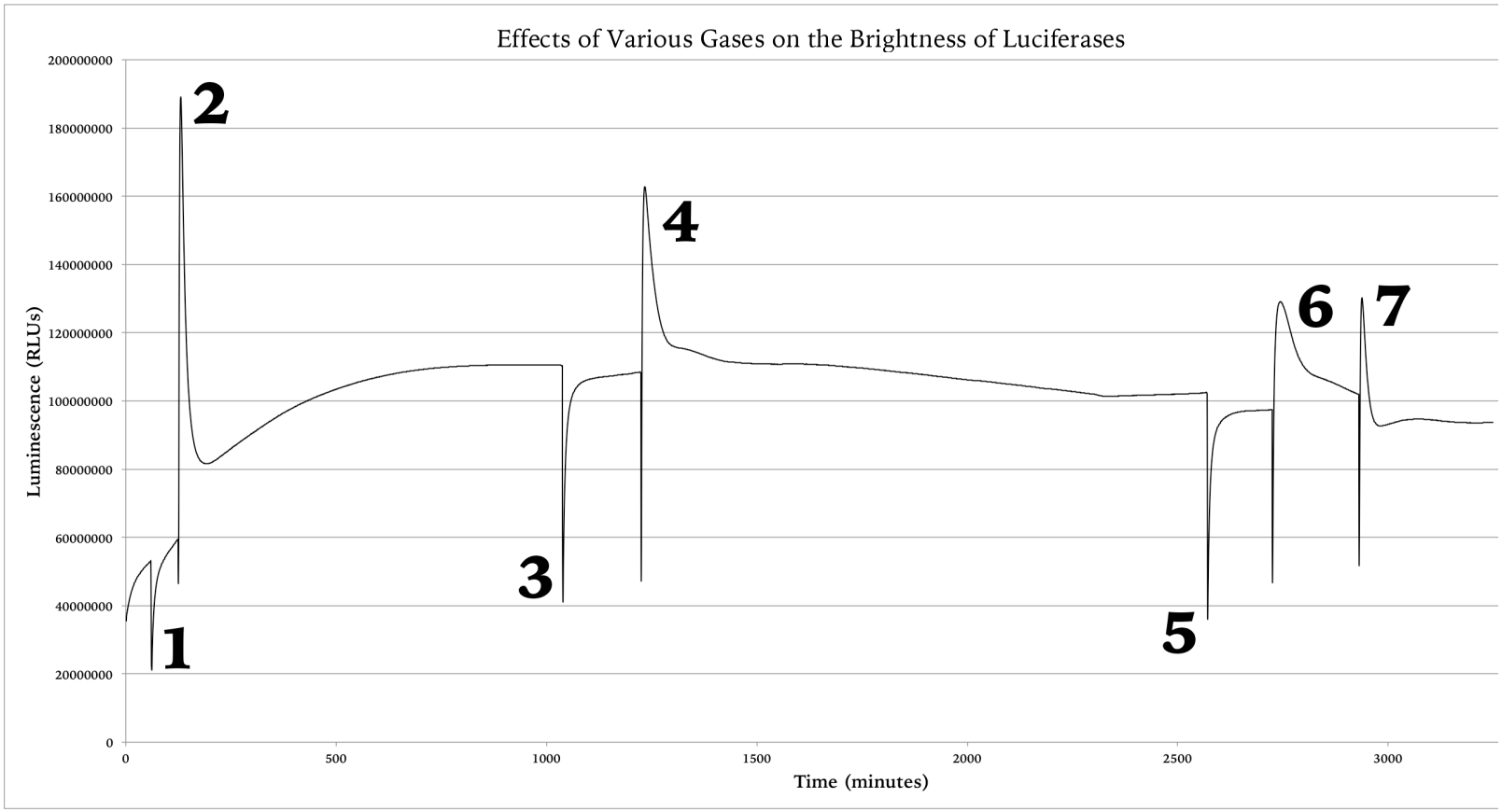

Figure 3: Effects of Various Gases on the Brightness of Luciferases – Bacterial luciferase with HoxABCJN1. Points 1, 3, and 5 mark the addition of air to the culture. Points 2 and 6 mark the addition of hydrogen to the culture. Points 4 and 7 mark the addition of oxygen to the culture. All points show a brief dip before returning to normal levels or increasing. Air dips before returning to normal while hydrogen and oxygen both lead to an increase in brightness. Hydrogen leads to an increase that is comparable to or greater than that of oxygen.

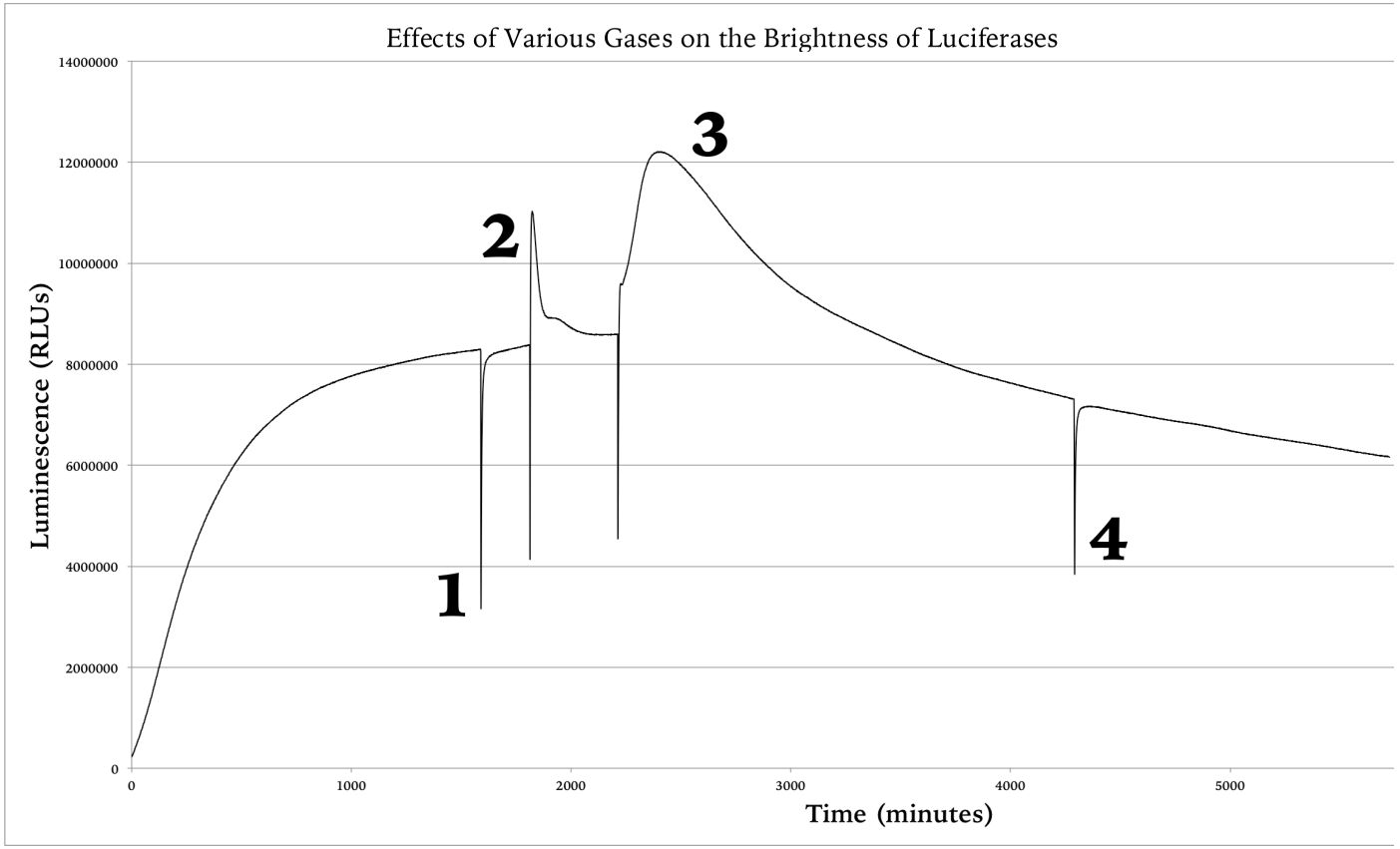

Figure 3 (cont.): Effects of Various Gases on the Brightness of Luciferases – Δ*hoxJ* bacterial. Points 1 and 4 mark the addition of air to the culture, while points 2 and 3 mark the addition of hydrogen and oxygen, respectively. The increase after addition of hydrogen is again comparable to the increase after the addition of oxygen. Since these *E. coli* do not have any of the hydrogen sensing machinery, this must be due to sensitivity of the luciferase itself to hydrogen.

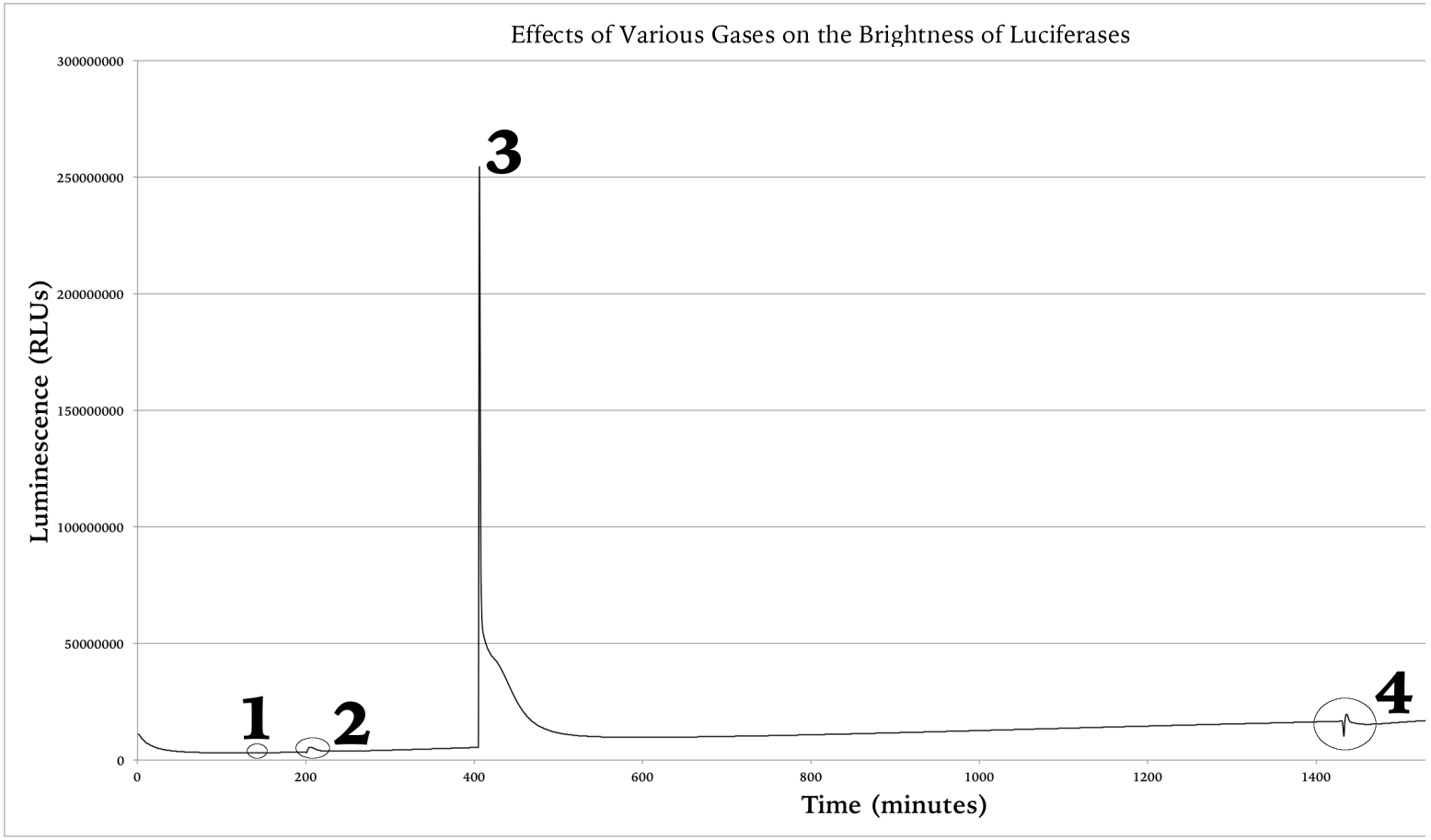

Figure 3 (cont.): Effects of Various Gases on the Brightness of Luciferases – CBG99 with no Hox machinery. Points 1, 2, 3, and 4 mark the addition of air, hydrogen, oxygen, and helium, respectively. Exposure to hydrogen caused a small increase in luminescence, but exposure to oxygen caused a much larger increase. CBG99 does not demonstrate the same hydrogen sensitivity as bacterial luciferase.

P_{MBH} Drives Transcription in *E. coli*

E. coli transformed with the CBG99 reporter constructed for *R. eutropha* (CBG99-pBBR1MCS-2) were compared to *E. coli* transformed with unmodified pBBR1MCS-2. CBG99-pBBR1MCS-2 contains CBG99 driven by P_{MBH} but no *hoxA* gene. As shown in Figure 4, the *E. coli* containing CBG99-pBBR1MCS-2 are glowing while those containing only pBBR1MCS-2 are not, meaning *E. coli* can recognize P_{MBH} to some extent.

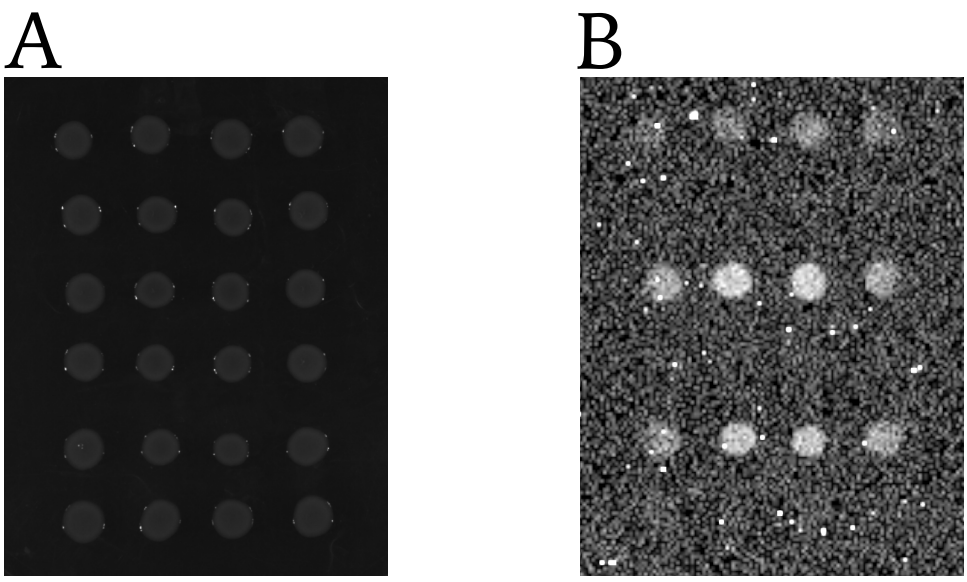

Figure 4: P_{MBH} Drives Transcription in *E. coli* – A) Brightfield image of *E. coli* transformed with CBG99-pBBR1MCS-2 or unmodified pBBR1MCS-2 patched on LB agar. B) Rows of cells transformed with CBG99-pBBR1MCS-2 are glowing, indicating recognition of P_{MBH} by *E. coli*, while cells transformed with unmodified pBBR1MCS-2 are not glowing.

HoxJ Inhibits HoxA in *E. coli*

E. coli transformed with Δ*hoxJ*-pTrc99A or *hox*-pTrc99A were patched in an alternating pattern on an LB agar plate containing IPTG. This results in the production of the Hox proteins since those genes are under the control of the *trc* promoter. As shown in Figure 5, the two strains are clearly distinguishable from one another. This demonstrates that the HoxJ protein can inhibit HoxA in *E. coli* to stop it from starting transcription at P_{MBH}.

hox-pTrc99A in *E. coli*

E. coli with Δ*hoxJ*-pTrc99A or *hox*-pTrc99A were grown on LB agar in flasks and exposed to hydrogen. Cells in a second flask were not exposed to hydrogen as a control. The exposure to hydrogen was carried out in a variety of ways, all with the same result. First, the cells were grown overnight at 37°C. Then, 50mL H_2 was injected and the flask stoppered. The H_2 was left in the flask for varying amounts of time, ranging from 5-15 minutes, to 1-2 hours, to overnight. The experimental and control flasks were both imaged and the relative brightness levels of the Δ*hoxJ*-pTrc99A and *hox*-pTrc99A strains within each flask were compared. Other times, cells were grown overnight at 37°C with hydrogen in the flask. 50mL of H_2 was injected once at the start of incubation or every 4 hours until the flasks were imaged. In all cases, the Δ*hoxJ*-pTrc99A and *hox*-pTrc99A strains were grown in successive batch cultures until they reached

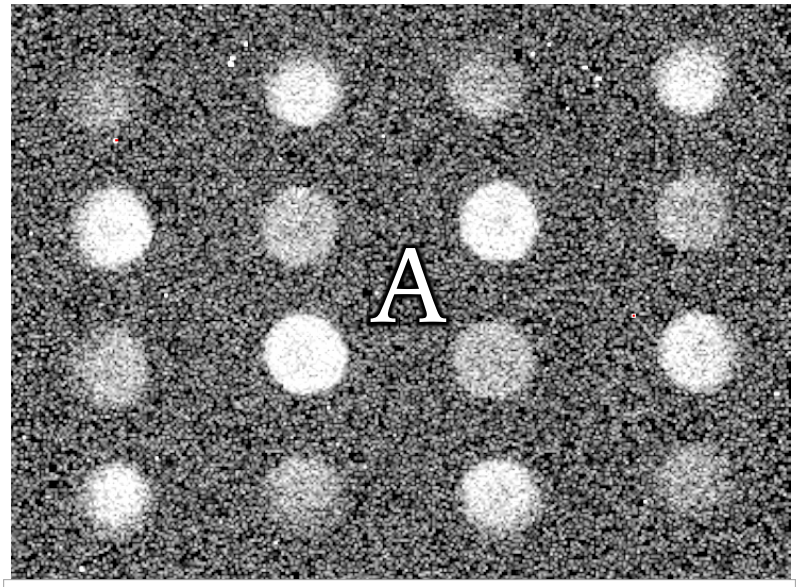

Figure 5b

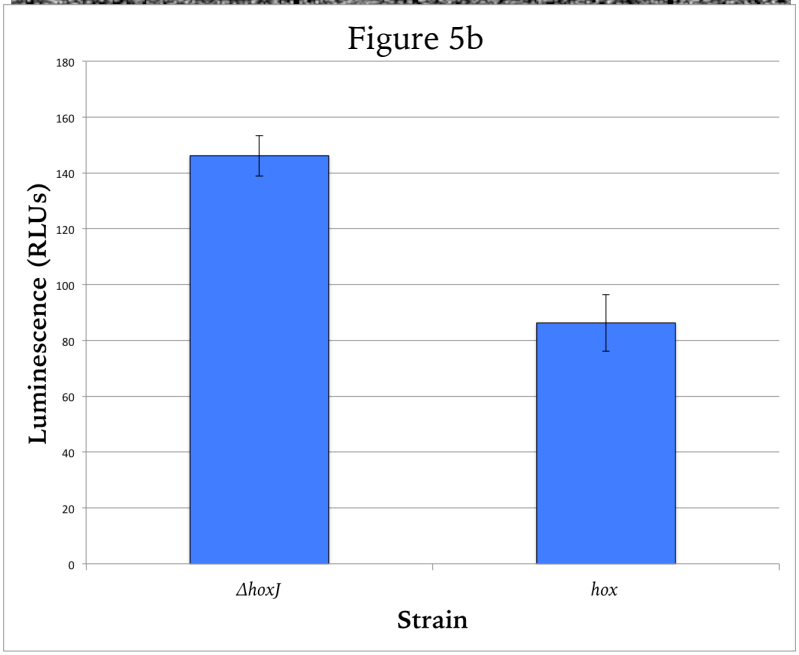

Figure 5: HoxJ Inhibits HoxA in *E. coli* – A) Cells were patched on LB agar containing IPTG in alternating order with Δ*hoxJ*-pTrc99A or *hox*-pTrc99A. Patches containing Δ*hoxJ*-pTrc99A are visibly brighter than those containing *hox*-pTrc99A, indicating that HoxJ is inactivating HoxA when present. B)Graph of brightness levels measured in ImageJ. Error bars represent 1 standard deviation.

the same OD (as described in Materials and Methods) to control for cell number. Figure 6 shows a typical result, where the *hox*-pTrc99A strain is the same relative brightness compared to the Δ*hoxJ*-pTrc99A strain as when no hydrogen was injected. If the *hox*-pTrc99A strain were properly folding the $Hox(BC)_2$ protein, which would split H_2 and inactivate HoxJ, one would expect those cells to be as bright as the Δ*hoxJ*-pTrc99A cells that have *hoxJ* deleted. Since it has been established that HoxA is recognizing P_{MBH} to start transcription, and that HoxJ is inactivating HoxA when present, the problem may lie in the ability of $Hox(BC)_2$ to split hydrogen or inactivate HoxJ. After the release of two papers in 2013 (see Introduction) stating that the accessory Hyp proteins were necessary for the production of catalytically active soluble hydrogenase (SH) from *R. eutropha* in *E. coli*, the *hyp*-pACYC184 plasmid was constructed to provide the proper *hyp* genes to fold $Hox(BC)_2$ correctly.

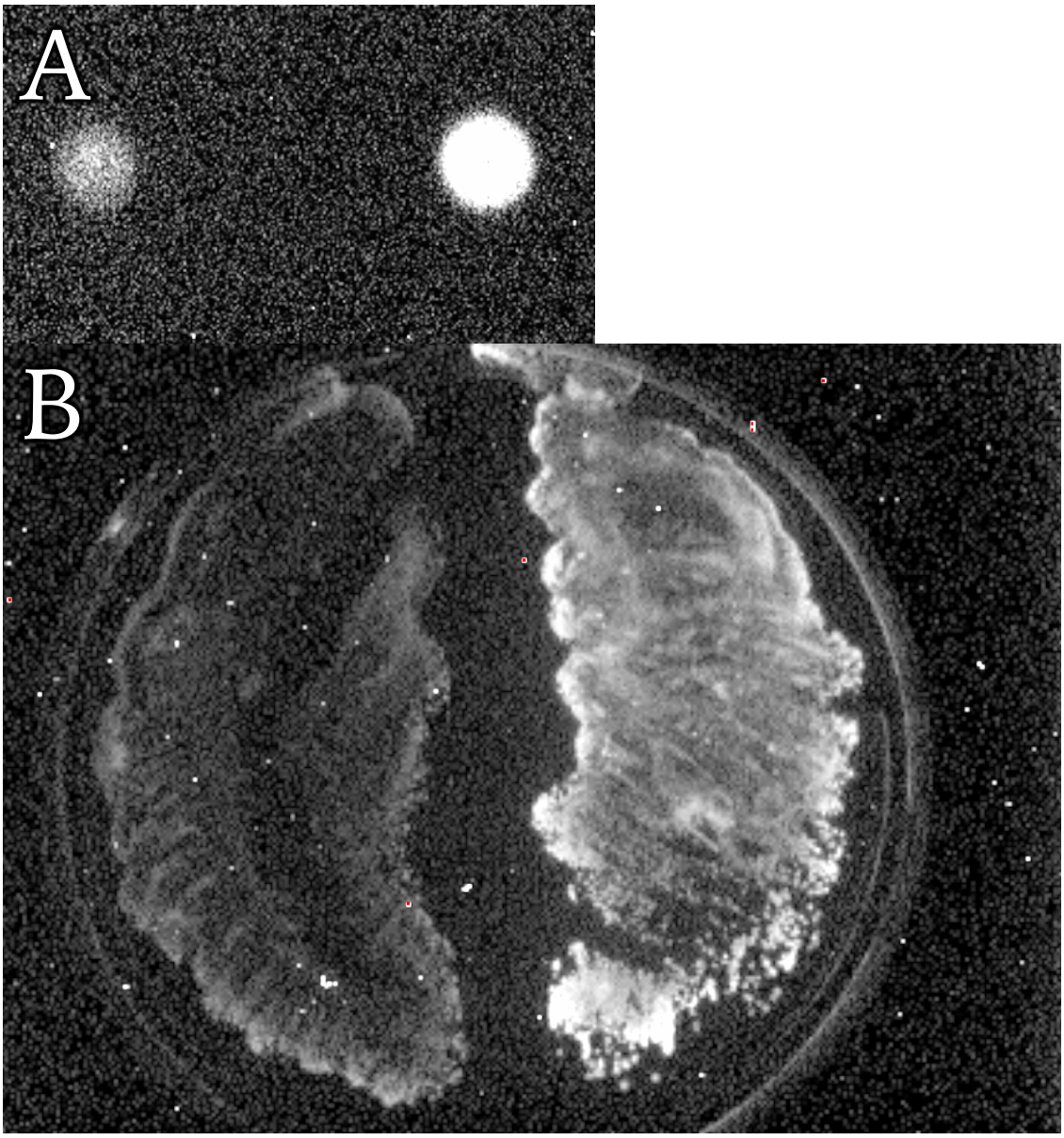

Figure 6: *hox*-pTrc99A Does Not Confer Hydrogen-Sensitive Glowing in *E. coli* – A) *hox E. coli* (left) are much dimmer than Δ*hoxJ E. coli* (right) after being grown in the presence of hydrogen. If the cells responded to the presence of hydrogen, the patch on the left should be closer in brightness to the patch on the right. B) The two strains were streaked onto the bottom of a flask rather than pipetted in precise amounts. The same relative brightness levels are observed.

hyp-pACYC184 and *hox*-pTrc99A in *E. coli*

E. coli containing Δ*hoxJ*-pTrc99A or *hox*-pTrc99A were made competent and transformed with *hyp*-pACYC184. Double transformants were grown on agar in flasks in the same manner as the single transformants. Flasks were injected with 50mL H_2 and incubated at 37°C overnight. When imaged, the two strains showed the same relative levels of brightness as when they didn't contain *hyp*-pACYC184 (Figure 7), though they were dimmer overall than the single transformants and grew more slowly. This would imply that either: the Hyp proteins encoded by the MBH operon are not sufficient to properly fold the hydrogen sensor in *E. coli*; or something other than HoxABCJN1 is needed to confer hydrogen-sensing ability to *E. coli*. Since the Hyp proteins encoded by the SH operon were successfully used to produce catalytically active SH in *E. coli*, the first potential reason seems somewhat unlikely. As reviewed in the Introduction to this report, there is more that controls hydrogenase expression in *R. eutropha* than the presence or absence of hydrogen. The presence of certain carbon sources influences hydrogenase expression, though the mechanism through which this happens is not understood.

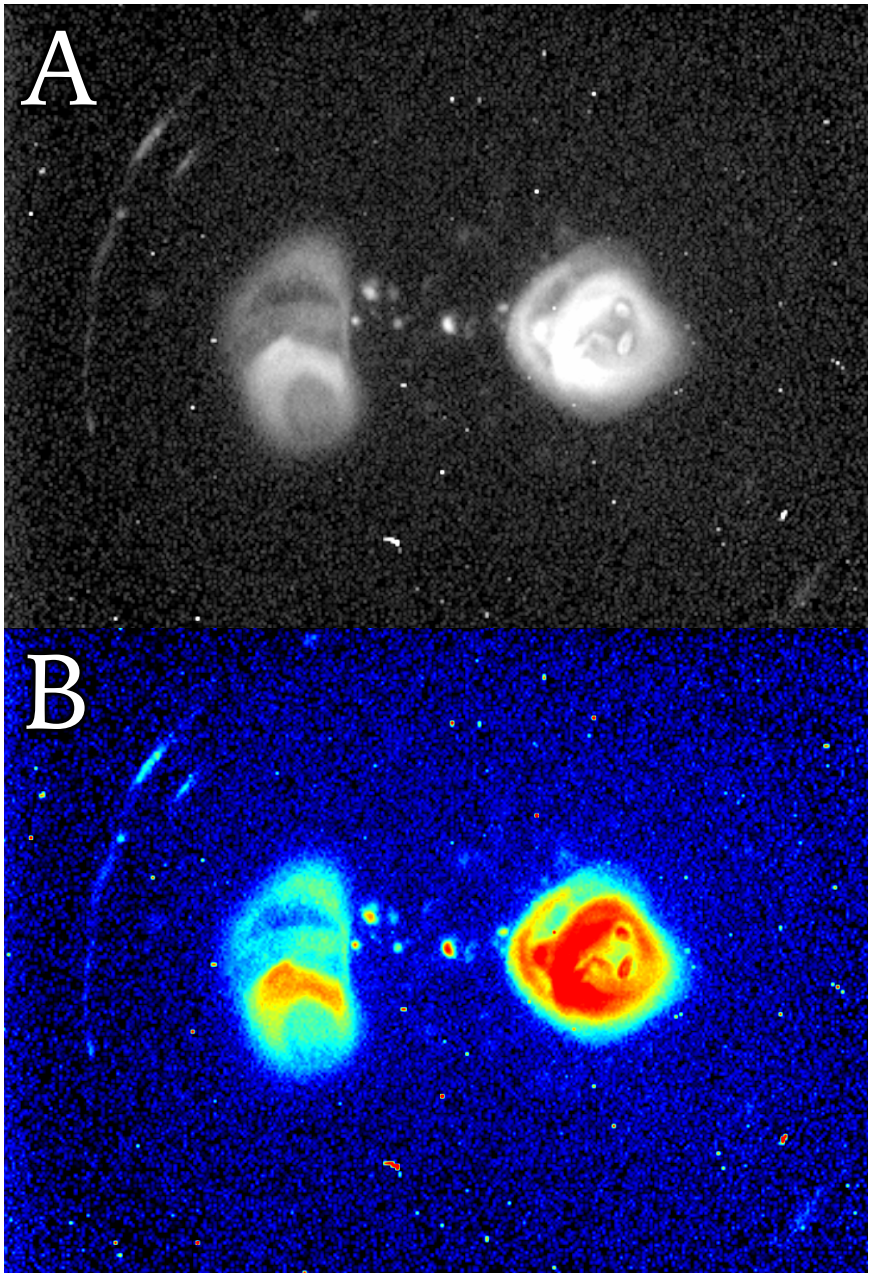

Figure 7: The Addition of *hyp*-pACYC184 Does Not Confer Hydrogen-Sensitive Glowing in *hox*-pTrc99A *E. coli* – *hox-hyp E. coli* (left) are dimmer than Δ*hoxJ-hyp E. coli* (right) after being grown in the presence of hydrogen. A) is a black-and-white image while B) has color values assigned by the BioRad ImageLab software to represent brightness. Colors follow ROYGBIV order with red being the brightest.

Succinate Represses *R. eutropha* Hydrogenase Expression

Lenz, *et al*. (2002) demonstrated with a β-galactosidase reporter that hydrogenase expression in *R. eutropha* is tightly repressed when grown on succinate and derepressed when grown on fructose or glycerol. Those results were reproduced for this report using *R. eutropha* containing the CBG-pBBR1MCS-2 reporter (Figure 8). Cells were grown overnight at 30°C in liquid SN media and placed in 5μL circles on SN and GN plates, which were incubated for 5 days. Succinate is the preferred carbon source for *R. eutropha,* so the cells on the SN plate grew faster; the patches were noticeably thicker than on the GN plate after 5 days. Despite the smaller cell number, however, the cells on the GN plate were considerably brighter. As this replicates the results from previous studies, it further supports that the P_{MBH}-CBG reporter accurately reports hydrogenase expression in *R. eutropha*. This is important since the presence of hydrogen does not seem to affect the brightness levels of *E. coli* containing *hox*-pTrc99A and *hyp*-pACYC184. Since the reporter works properly in *R. eutropha,* this would imply that it is, in fact, the hydrogen-sensing system that is not working in *E. coli* and not the reporter itself.

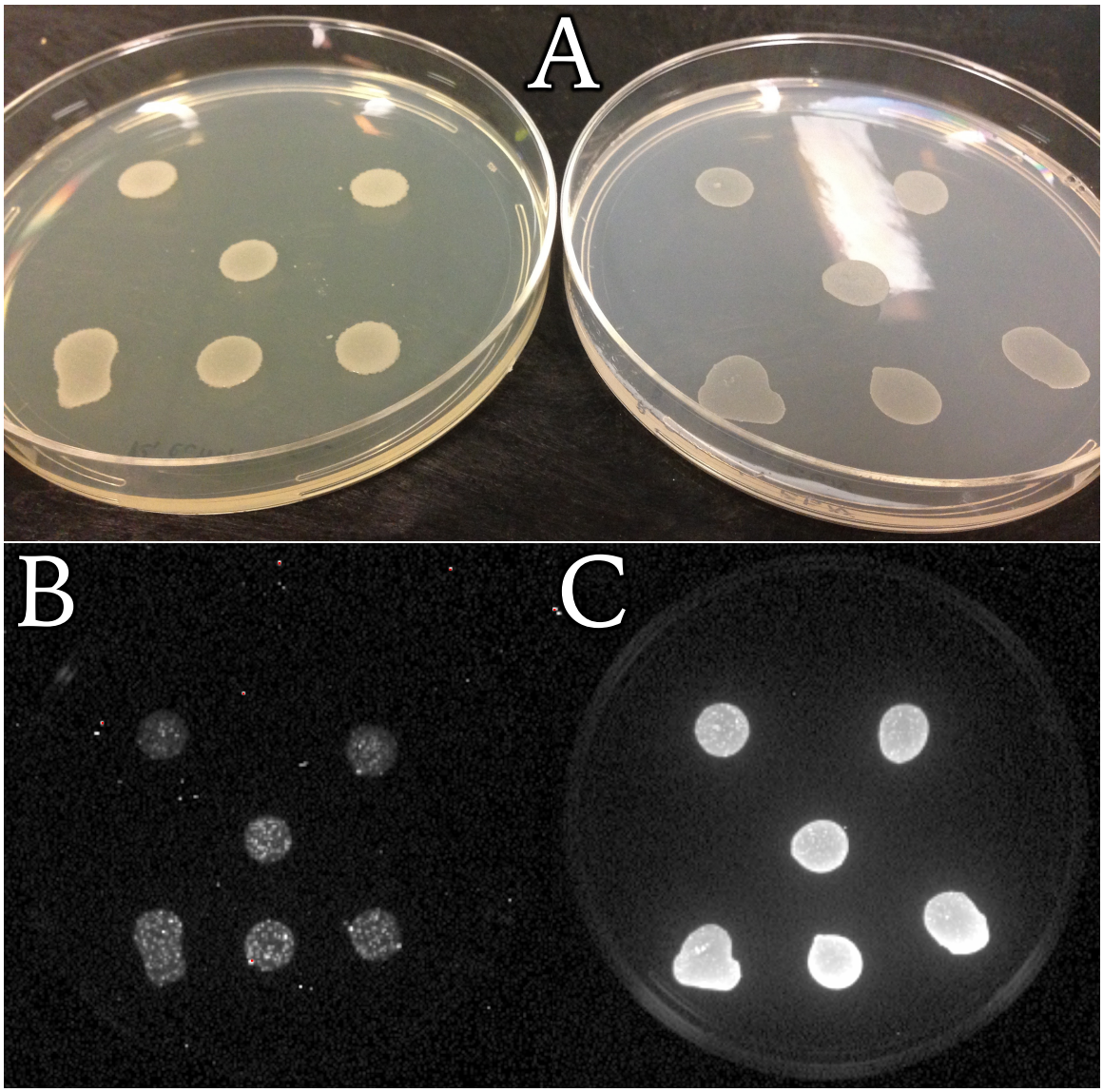

Figure 8: Hydrogenase Expression in *R. eutropha* is Repressed by Growth on Succinate – A) Cells on SN (left) are visibly thicker than cells on GN (right) after 5 days. SN is the preferred carbon source. B) Cells on SN plate imaged with a 5 minute exposure. C) Cells on GN imaged with a 20 second exposure. Despite being fewer in number, these cells are considerably brighter than the cells growing on SN, indicating that the hydrogenase genes are greatly upregulated in these cells.

R. eutropha Can Use Delivered Hydrogen

Cells grown on GN medium in a stoppered flask with hydrogen grow faster than cells grown on just GN medium (figure not shown). Additionally, *R. eutropha* spread on a mineral salts agar containing no carbon source will not grow unless supplied with hydrogen and CO_2 (figure not shown). This demonstrates that the method of hydrogen delivery used throughout the experiments in this report is viable. If the stoppered flasks were unable to hold the hydrogen for long enough to be detected by the bacteria, *R. eutropha* would not be able to grow using this energy source. Furthermore, hydrogen can still be popped from the flasks after incubating with the cells overnight, so the stoppers are at least holding a fair amount in.

CHAPTER FOUR: Conclusions

The goal of this project, as previously stated in the Introduction, was to examine the viability of combining *R. eutropha*'s hydrogen-sensing pathway with a luciferase to create a biological hydrogen-sensing reporter for heterologous expression. Based on the results presented in this report, this pathway is not suitable for creating a heterologous hydrogen-sensing reporter. While the P_{MBH}-CBG99 combination appears to be a good reporter of hydrogenase expression in *R. eutropha*, the pathway itself is not well enough understood for full heterologous expression in *E. coli* or an even more distantly related species like cyanobacteria or green microalgae.

Since *E. coli* containing *hox*-pTrc99A and *hyp*-pACYC184 do not glow in a hydrogen-responsive manner, it could be that something else from *R. eutropha* is required for the system to work correctly in *E. coli*. This is complicated by the fact that hydrogen is not the only factor that influences hydrogenase production in *R. eutropha*. The presence of different carbon sources plays a role in the regulation of hydrogenase production; however, it's not understood exactly how this happens, only that it does. Preferred carbon sources like succinate will repress the expression of hydrogenases, even in the presence of hydrogen, while poorer sources such as glycerol allow for hydrogen-responsive gene regulation.

Depending on how this works, it may or may not be the reason behind the system's failing to work properly. If the presence of succinate leads to a metabolite that directly represses HoxA or blocks P_{MBH}, this would presumably not be a problem in *E. coli* since that part of the system was not transferred on the constructed plasmids. However, the system could work similarly to the *lac* system in *E. coli*, where the presence of lactose does not guarantee high-level production of β-galactosidase. In this system, the presence of lactose removes the lacI repressor from the equation (much like the presence of hydrogen leads to the inactivation of HoxJ), but the presence of a preferred carbon source (glucose) results in low cAMP levels in the cell. Binding of cAMP to CAP is required for maximum β-galactosidase production, so low cAMP levels as a result of the presence of glucose leads to relatively smaller β-galactosidase expression. While this is not perfectly analogous to the hydrogen-sensing pathway, it could be possible that a similar system is required to fully activate hydrogenase production. This system would not have been transferred to *E. coli* on the constructed plasmids so activation of P_{MBH} would remain low. However, since cells lacking *hoxJ* are brighter, this would imply that this theoretical system would be necessary for the inactivation of HoxJ. It could be that the unidentified redox cofactor bound to HoxJ is only produced in high levels when

succinate is absent from the cell, much like cAMP levels in *E. coli* are only high when glucose is absent from the cell.

Another possibility for the lack of hydrogen-responsive glowing is that the Hox$(BC)_2$ protein is still not being assembled correctly in *E. coli*, despite being provided with the *hyp* accessory proteins needed for proper folding in *R. eutropha*. The soluble hydrogenase (SH) from *R. eutropha* has been expressed in *E. coli* in a catalytically active form by co-expression with (different) Hyp proteins, but the genes for the SH and those Hyp proteins are found in a different operon. It could be that something else entirely that is present in *R. eutropha* and not *E. coli* is necessary. While this doesn't seem incredibly likely, it is a possibility nonetheless.

Overall, *E. coli* using P_{MBH} to drive CBG99 are very dim compared to *R. eutropha* with the same system. At their brightest, *E. coli* need around a 10 minute exposure to be seen well, with HoxA being produced under *trc* control and no HoxJ to inhibit it. *R. eutropha*, on the other hand, need only a few seconds to saturate the detector when grown on GN medium with no exposure to hydrogen. It seems that *E. coli* is generally not very good at recognizing P_{MBH} to start transcription, even with HoxA being produced in large quantities. Since transcription in *R. eutropha* is dependent on that organism's σ-54 sigma factor, it

may be that *E. coli*'s σ-54 only very weakly recognizes P_{MBH}. Whatever the case, the failure of *E. coli* to be able to fully turn on expression at P_{MBH} when producing unrepressed HoxA presents the biggest challenge in this research. Even if *E. coli* containing *hox*-pTrc99A glowed in a hydrogen-dependent manner, the dimness of the glowing provides a very narrow window to actually see results. Δ*hoxJ*-pTrc99A *E. coli* can't be seen with a 10 minute exposure when grown at 30°C or on minimal media such as M9. Furthermore, there's also a relatively narrow window of cell activity during which the glowing is visible, and then only when grown on rich media at 37°C. On the other hand, *R. eutropha* grown on a GN plate are visible with an exposure of a few seconds after sitting at room temperature for a month. Whether the result of *E. coli*'s RNA polymerase, σ-54, or some yet unidentified factor, this system is unsuited for use in a heterologous hydrogen-sensing reporter unless this problem is solved.

R. eutropha made a good hydrogen-sensing organism for primary investigations because of the body of basic research conducted on it. It is certainly not the only hydrogen-sensing bacterium, but *R. eutropha* has multiple biotechnological applications that have led to more interest in it than in other organisms. For future research, it might be a good idea to focus on an organism that regulates gene expression solely in response to hydrogen presence, not also

some other factor such as presence of various carbon sources. There is one such organism that is very closely related to *R. eutropha* H16 (ATCC 17699). When H16 was classified as *Alcaligenes eutrophus,* the organism filed as ATCC 33178 was classified as *Alcaligenes hydrogenophilus*. They are now both classified as *Cupriavidus necator*. However, while using such a closely related organism would no doubt provide some advantages considering the greater amount of research on *R. eutropha* H16, the problem of low expression from a promoter that *A. hydrogenophilus*'s HoxA would bind to would almost certainly remain. There are other bacteria that also regulate some kind of gene expression based solely on the presence of hydrogen, such as *Bradyrhizobium japonicum* or *Rhodobacter capsulatus,* but not as much research has been done on them as on *R. eutropha*. There is no guarantee that the dimness problem would be solved by switching to one of these other bacteria. Unfortunately, most of the research on bacterial hydrogen sensors was done in the late 1990s and early 2000s. *R. eutropha*'s resistance to heavy metals and ability to produce polyhydroxybutyrate has driven interest in that organism. In recent years, basic research appears to have shifted away from hydrogen sensors and toward biotechnological applications of other hydrogenases, despite the fact that some mysteries surrounding the sensors,

such as how carbon sources factor into hydrogenase gene expression in *R. eutropha*, remain.

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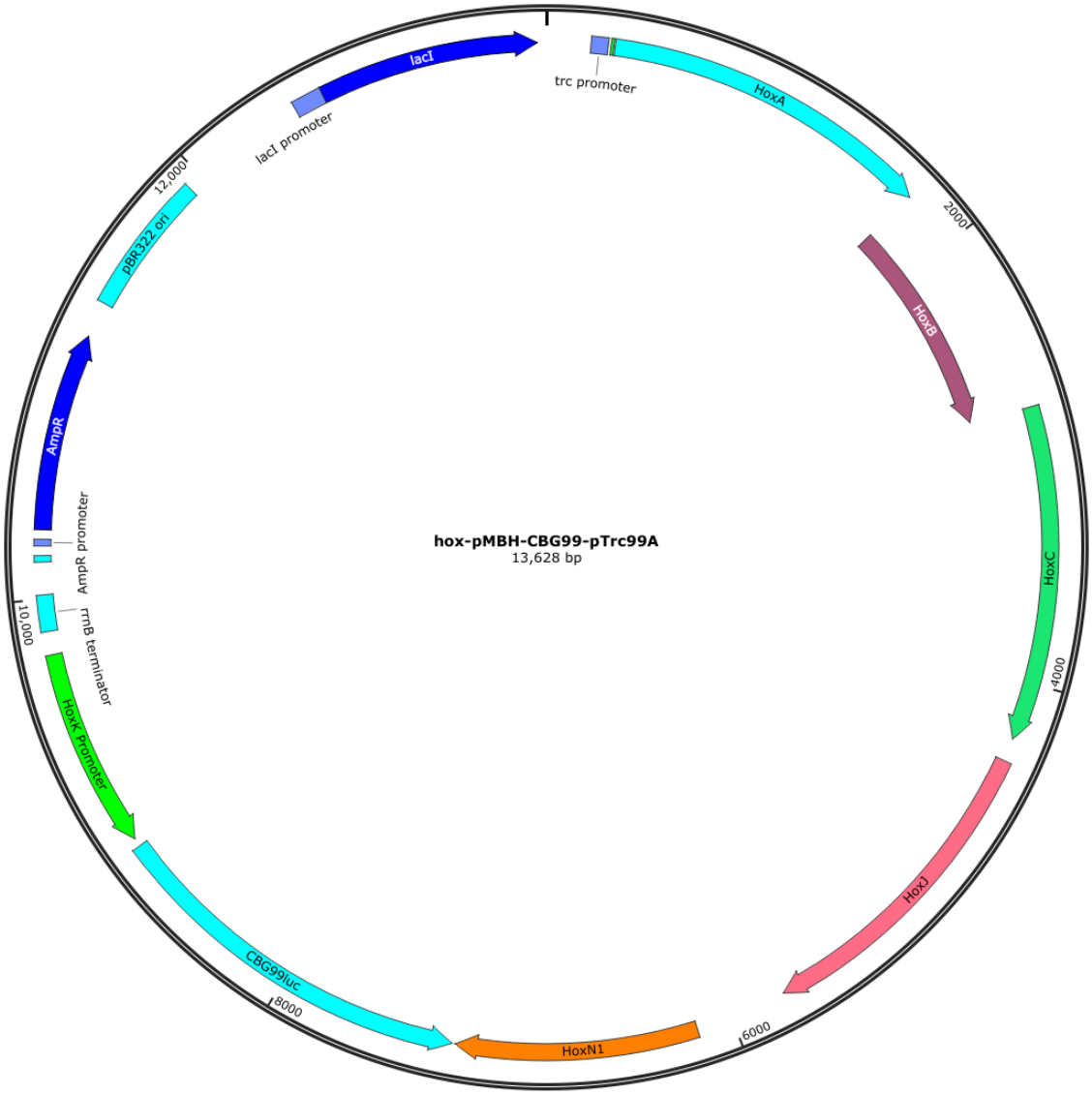
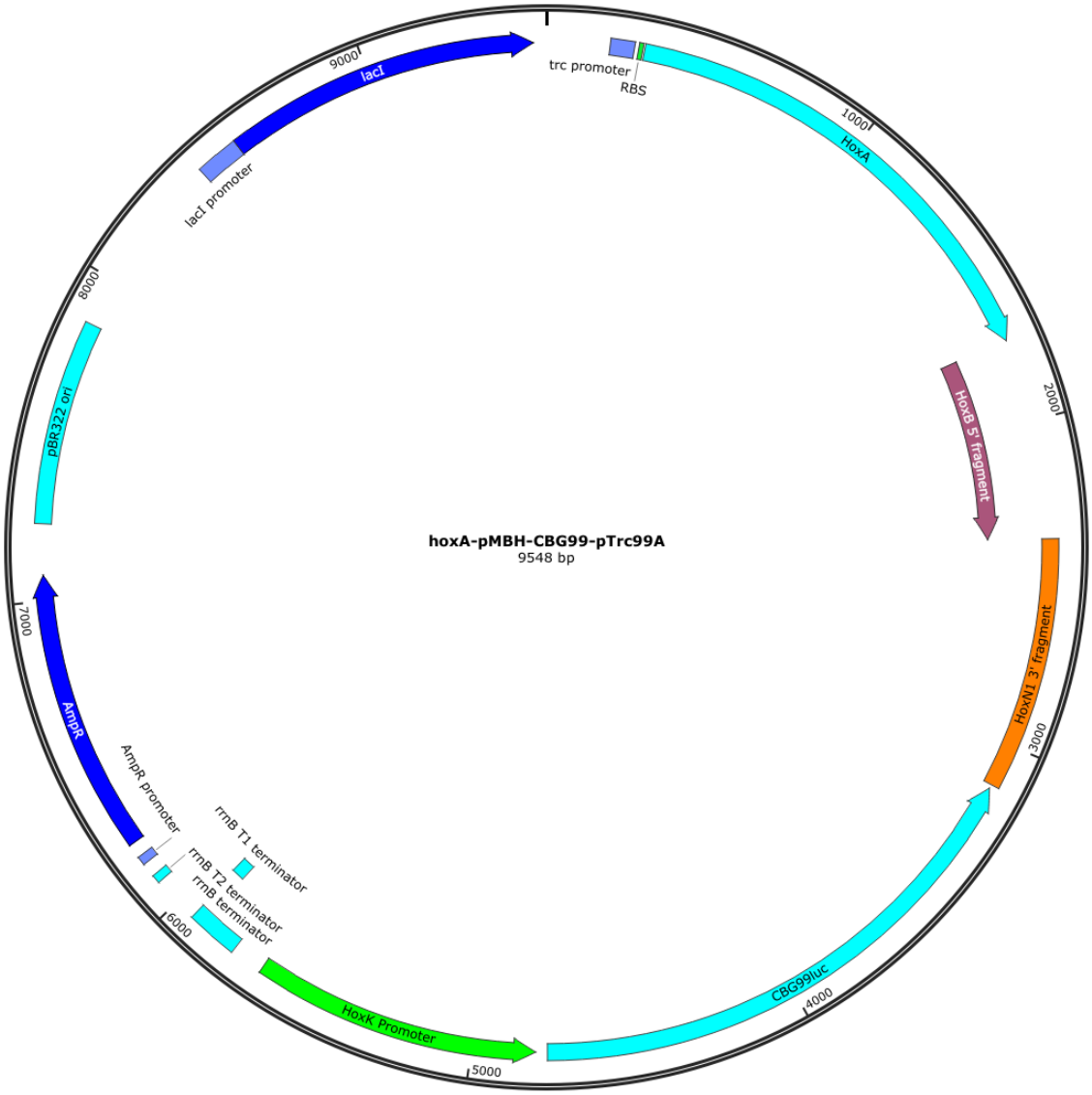
Appendix A: Supplementary Material

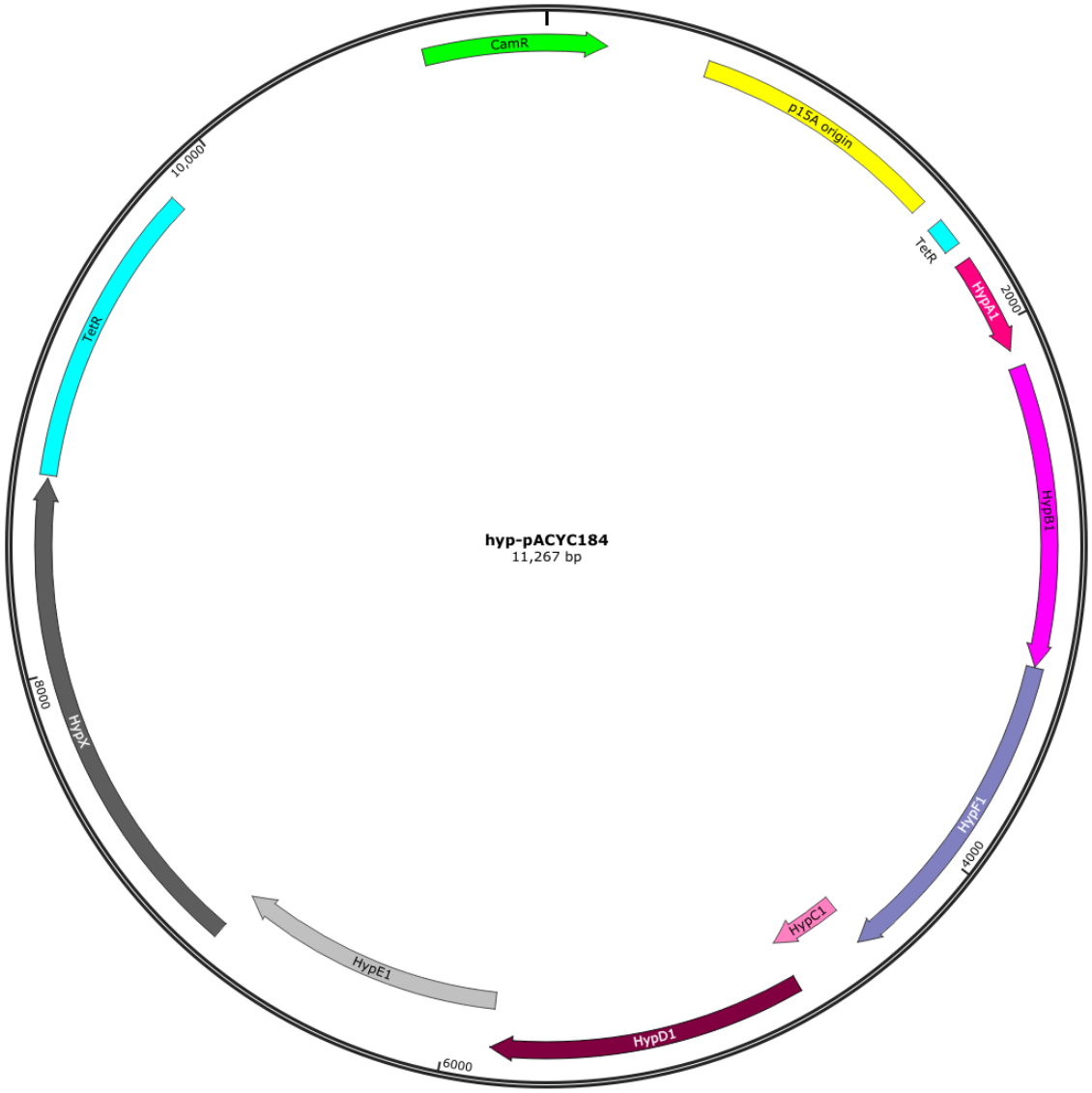
Primers

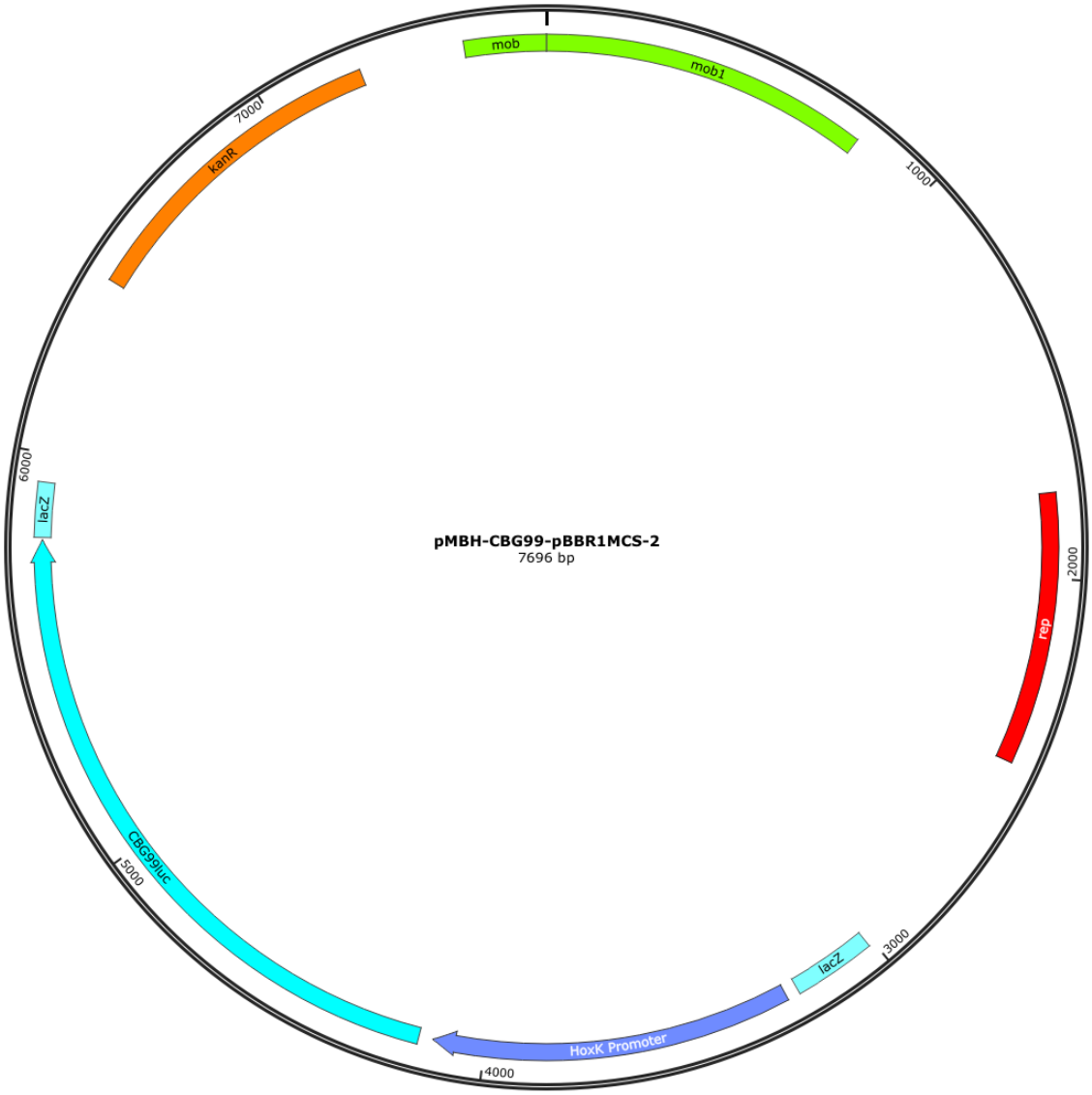
Sequences for DNA hybridization are underlined. Restriction sites are red. In Primer 16, the *tac* promoter is blue.

#	Sequence	Name
1	aagtaaCTCGAGTTATCACTGCACACGCTGTC	HoxK promoter 5
2	aagtaaTCTAGATAACCTGTCTCCTAATTTCTGT	HoxK promoter 3
3	aagtaaGGATTCATGTCTGACAAGCAGGCCAC	HoxA start 5 (BamHI)
4	aagtaaGGATTCTCAGGCACGCACTTCGCTG	HoxN1 end 3 (BamHI)
5	TGGCGCACGAGTTGAACAAT	HoxJ AflII site 5
6	aagtaaGGATCCTCAGGCACGCACTTCGCTG	HoxN1 end 3 (BamHI)
7	CAGACCATGGAATTCGAGCTC	Cloning site screen 5
8	GTGGGCTGGCGTAATGAATG	HoxA screen 3
9	aagtaaCTCGAGTTATCACTGCACACGCTGTC	HoxK promoter 5 (XhoI)
10	aagtaaAAGCTTTAACCTGTCTCCTAATTTCTGT	HoxK promoter 3 (HinDIII)
11	GTTTTCTGGAACGCGGTTGG	pTrc-LuxAB screen 5
12	GTGGGCTGGCGTAATGAATG	HoxA screen 3
13	atttatCCTGCAGGGCACACGCTGTCGCTGGAAT	HoxK promoter 5 (SbfI)
14	agtggtCCTGCAGGtctagaCTAACCGCCGGCCT	CBG99 3 (SbfI)
15	TTGAAGCTACCAAGGAGGCC	pTrc-CBG99 screen 3
16	atcccgTCTAGAAAGCTTGTTGACAATTAATCATCGGCTCGTATA ATGTGTGCGTTAAAAGGAGATATAATGCATGAGCTCAGCCTGGC	HypA1 start 5 & pTac (XbaI & HinDIII)
17	aatgatTGATCATCTAGACATGGCTCAAGATCGTTTCC	HypX end 3 (XbaI & BclI)
18	atatgtGAGCTCGTGATGGTGAAGCGTGAGAAAAA	CBG99 start 5 (SacI)
19	tctatatatGAGCTCCTAACCGCCGGCCTTCTCCA	CBG99 end 3 (SacI)
20	TCGCCTTCGACTGCTATAA	HypX screen 5


hox-pMBH-CBG99-pTrc99A
13,628 bp
lacI
lacI promoter
trc promoter
HoxA
HoxB
HoxC
HoxJ
HoxN1
CBG99luc
HoxK promoter
rrnB terminator
AmpR promoter
AmpR
pBR322 ori
2000
4000
6000
8000
10,000
12,000


hoxA-pMBH-CBG99-pTrc99A
9548 bp
9000
lacI
lacI promoter
trc promoter
RBS
1000
HoxA
2000
HoxB 5' fragment
HoxN1 3' fragment
3000
CBG99luc
4000
HoxK Promoter
5000
rrnB T1 terminator
rrnB T2 terminator
rrnB terminator
6000
AmpR promoter
AmpR
7000
pBR322 ori
8000


CamR
p15A origin
TetR
HypA1
HypB1
HypF1
HypC1
HypD1
HypE1
HypX
TetR
10,000
2000
4000
6000
8000
hyp-pACYC184
11,267 bp


mob
mob1
1000
rep
2000
lacZ
3000
HoxK Promoter
4000
CBG99luc
5000
lacZ
6000
kanR
7000
pMBH-CBG99-pBBR1MCS-2
7696 bp

Composition of FGN Minimal Medium:

Stock Solutions:

- 10x H16 Buffer (35.67 g Na_2HPO_4 or 67.36 g for heptahydrate, 15 g KH_2PO_4. q.s. to 1 L ddH_2O, titrate to pH 7.0 with phosphoric acid, if necessary)
- 20% (w/v) NH_4Cl; autoclave!
- 20% (w/v) $MgSO_4$ x 7 H_2O; autoclave!
- 1% (w/v) $CaCl_2$ x 2 H_2O; autoclave!
- 0.5% (w/v) $FeCl_3$ x 6 H_2O (in 0.1 N HCl); filter!
- 1mM (w/v) $NiCl_2$; autoclave!
- 40% (w/v) fructose; filter!
- 40% (w/v) glycerol; autoclave!

Composition for 1 liter liquid $F_{0.05}G_{0.4}N$ medium:

Mix 100 mL 10x H16 buffer with 875 mL H_2O and autoclave. The following abovementioned sterile stock solutions must be added after sterilization:

- 10 mL NH_4Cl
- 1 mL $MgSO_4$ x 7 H_2O
- 1 mL $CaCl_2$ x 2 H_2O
- 1 mL $FeCl_3$ x 6 H_2O
- 1mL $NiCl_2$
- 1.25 mL fructose
- 10 mL glycerol